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van der Wildt, B.

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Chapter 6

Summary, general discussion and future perspectives

6.1 Summary

Transglutaminases are enzymes that are well recognized for their calcium dependent intermolecular crosslinking of protein substrates. The newly formed isopeptide or epsilon-(gamma-glutamyl)lysine bond between the former glutamine and lysine residue of the respective substrates is highly resistant to proteolysis. The resulting oligomeric protein structures introduce tissue rigidity and resistance to mechanical stress, which is important in for example skin formation and blood clotting.

Transglutaminase type 2, TG2, plays a role during cellular apoptosis, where crosslinking of intracellular components prevents leakage of these components out of the cells whilst these cells are being degraded, thus preventing inflammatory responses. The crosslinking of TG2 is tightly regulated. High guanosine diphosphate and triphosphate levels hold the enzyme in its closed inactive conformation. High calcium concentrations, in the millimolar range, allow the enzyme to adapt an open conformation, exposing the active site and thus enabling crosslinking. Further transglutaminase activity is controlled by an intramolecular disulfide bond, which must be reduced for transglutaminase activity.

Despite the tight regulation of TG2 crosslinking activity, excessive crosslinking activity of TG2 has been implicated in the onset of various seemingly unrelated diseases, including neurodegenerative diseases, fibrotic diseases, cancer and celiac disease. As a result, various inhibitors of TG2 have been developed, ranging from substrates, reversible and irreversible active site small molecule inhibitors, allosteric small molecule inhibitors, to TG2 inhibiting antibodies. Although some of these compounds seem highly promising *in vitro*, the actual target engagement of these compounds in living systems, *i.e.* test animals or human subjects, remains largely unknown. In addition, the exact role of TG2 in disease states is a topic of discussion, as extrapolating *in vitro* findings to mammalian *in vivo* biology is not trivial for this

particular enzyme. An obvious reason is the limited availability of chemical probes for monitoring the extent of TG2 inhibition *in vivo*. The aim of the studies described in this thesis was to develop a PET tracer for imaging active TG2 *in vivo*. To this end, promising TG2 inhibitors were selected from the literature and used as lead compounds towards imaging of active TG2 in a suitable animal model.

Chapter 1 provides a general introduction on the topics that are investigated in this thesis. The family of transglutaminases and their role under physiological conditions is introduced. The relation of TG2 and disease is discussed and the basic principles of PET imaging are addressed.

Chapter 2 provides an overview of possible strategies towards the development of a suitable PET TG2 tracer. As no validated PET tracers had been described at that, its content is somewhat suggestive. First, the use of either irreversible or reversible inhibitors as lead compounds is discussed. It is noted that reversible orthosteric as well as allosteric inhibitors with high affinity for TG2, a criterion for successful PET tracers, are not readily available. In contrast, various potent peptidic and non-peptidic irreversible inhibitors have been described and pharmacologically characterized. Furthermore, as enzymatic crosslinking proceeds via a two-step mechanism, involving both a glutamine donor substrate and an amine acceptor substrate, using radiolabelled TG2 specific substrates might be a promising strategy. Especially the use of TG2 specific TG2 glutamine donor substrates provides an interesting line of research, as this first step in the 'ping-pong' mechanism is substrate specific. Finally, antibodies that potentially allow for monitoring of either extracellular expression levels or active conformation TG2, depending on the antibody characteristics, are discussed. In conclusion, the use of potent irreversible inhibitors, selective acyl-donor substrates or antibodies is recommended in light of the currently available pharmaceutical toolbox.

In **Chapter 3** radiosynthesis and *in vitro* evaluation of a set of three carbon-11 labelled TG2 inhibitors are described (figure 1). These inhibitors, described recently by the Cure Huntington's Disease Initiative, carry an acryl amide warhead and thus allow for carbon-11 labelling by a palladium mediated aminocarbonylation reaction. First, the unlabelled compounds were synthesized and their inhibitory potencies evaluated. For compound **1**, the obtained IC_{50} value was approximately a factor 200 higher than the literature value, whereas the other values were comparable. Using the methodology mentioned above, all three carbon-11 labelled compounds were isolated in decay corrected yields of 42-49%, based on carbon-11 carbon monoxide. Compounds, obtained in 10% ethanol in saline solutions, were evaluated in healthy rats by means of organ distribution and blood metabolite analyses. Based on the superior metabolic stability of [^{11}C]**3**, this compound was selected for further *in vitro* evaluation by a TG2 binding assay and autoradiography on MDA-MB-231 tumour sections, derived from highly metastatic breast cancer. The excellent selectivity towards the active conformation of TG2 in the bindings assay and the highly specific and selective binding in the autoradiography experiments warrant further evaluation of this compound in animal models of TG2 overactivity.

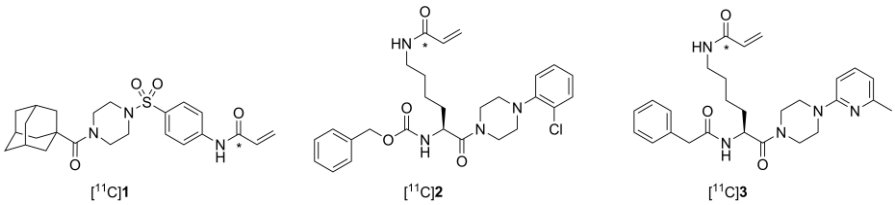

Figure 1: PET tracers [^{11}C]**1**-**3** described in **chapter 3**.

In **Chapter 4** the development of a TG2 PET tracer based on the well-known TG2 inhibitor **Z006** is described. **Z006** is a peptidic irreversible inhibitor of TG2 that carries a diazoketone warhead. Initially, attempts were made to perform carbon-11 labeling of **Z006** itself at the diazoketone carbonyl functionality, employing carbon-11 diazomethane. This is the common method for the introduction of diazoketones in

organic chemistry. However, in radiochemistry, where a large stoichiometric excess of precursor compared to the radioactive synthon is used, this method was unsuccessful. Instead, by replacing the N-terminal Z-group, eight analogues were synthesized that would allow for fluorine-18 labelling. Based on their *in vitro* inhibitory profile, two TG2 inhibitors, **6f** and **6g**, were selected for radiolabeling (figure 2). Radiolabelling of **6f** was performed in three steps by 1) fluorination of a tosylate methylester derivative of (*S*)-lactic acid precursor followed by distillation of the radiolabelled intermediate to a second reaction vial, 2) deprotection of the methyl ester and 3) BOP coupling to the corresponding precursor amine, resulting in [^{18}F]**6f** in an average 20% decay corrected yield. Radiolabeling of **6g** was performed in four steps in a one-pot reaction by 1) fluorination of the trimethylammoniumbenzaldehyde precursor, 2) oxime formation between the aldehyde and peptidic aminoxy-precursor, 3) conversion of the free carboxylic acid to a mixed isobutyl anhydride and 4) reaction of this anhydride with diazomethane towards the diazoketone, resulting in [^{18}F]**6g** in an average 9% decay corrected yield. Both compounds were evaluated in healthy rats by means of organ distribution and metabolite analyses. Compound [^{18}F]**6f** was rapidly converted to multiple metabolites, whereas compound [^{18}F]**6g** was converted to a single non-polar metabolite. Using LC-MS/MS analysis this metabolite [^{18}F]**M1** was identified as the C-terminally demethylated parent compound (figure 2). As this compound also is a strong TG2 inhibitor, further *in vitro* evaluation of [^{18}F]**6g** was performed.

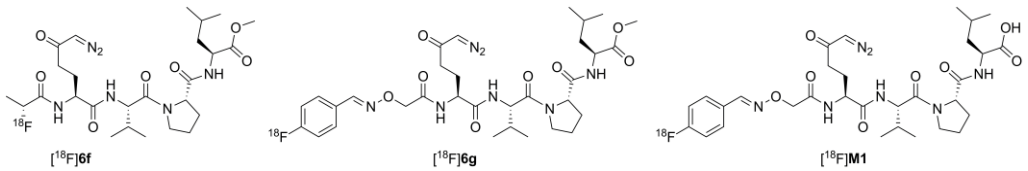

Figure 2: PET tracers [^{18}F]**6f**, [^{18}F]**6g** and metabolite [^{18}F]**M1** described in **chapter 4**.

Similar to the experiments in **Chapter 3** for compound [^{11}C]**3**, *in vitro* binding of [^{18}F]**6g** to isolated TG2 and autoradiography on MDA-MB-231 tumour sections demonstrated specific and selective binding to the active conformation of TG2. Based on these results, [^{18}F]**6g** should be evaluated in an appropriate animal model of TG2 overactivity.

In **Chapter 5** the *in vivo* evaluation of two promising TG2 radiotracers, developed in **Chapters 3** and **4**, using an orthotopic MDA-MB-231 tumour xenograft model in SCID mice is described as previous *in vitro* autoradiography experiments on MDA-MB-231 tumour sections demonstrated high amounts of TG2. Comparable metabolism of both tracers, [^{11}C]**3** and [^{18}F]**6g**, between SCID mice and the earlier used rats was observed. Importantly, similar metabolism of [^{18}F]**6g** towards the equipotent metabolite [^{18}F]**M1** was demonstrated. Tumours were inoculated at the second thoracic mammary fatpad, distant from organs that might disturb PET data analysis. PET scanning was performed with the two tracers at both baseline and blocking conditions using the TG2 inhibitor **ERW1041E**, an inhibitor that has been shown to inhibit TG2 *in vivo* in various animal models. Furthermore, co-administration of unlabelled **6g** was used for evaluation of [^{18}F]**6g** binding. Compound [^{11}C]**3** displayed low tumour targeting of approximately 0.2 %ID/g at the 40-60 minute timeframe, which was equal to background uptake, derived from muscle tissue of the hind leg. No increase of activity concentration was observed over time, suggesting lack of irreversible binding of the radiotracer. Furthermore, inhibition of TG2 by pretreatment of the mice with **ERW1041E** resulted in a counterintuitive increase in tumour activity concentrations, as well as in background tissue. In contrast, compound [^{18}F]**6g** did demonstrate a time-dependent increase in tumour activity concentrations up to 1.7 %ID/g at the 40-60 minute time-frame, compared with 0.8 %ID/g in background tissue. Pretreatment with **ERW1041E** resulted in a decrease in tumour activity concentrations to 1.4 %ID/g, although this was not considered statistically significant (p = 0.06). Blocking did not affect background uptake. Co-

administration of unlabelled **6g** resulted in a more drastic drop of tumour activity concentrations to 1.0 %ID/g at 40-60 minutes (p = 0.007). Post mortem examination of tumours sections by immunohistochemistry, histochemistry and autoradiography revealed that TG2 expression and activity were rather limited to the outer areas of the tumour tissue. This tightly corresponded with PET imaging data, where activity concentrations were lower towards the core of the tumours. In the core of tumours, fewer nuclei were present, as shown by haematoxylin staining, which would be in line with central tumour necrosis. In general, large parts of non-viable tissue would flatten out the actual potential of [^{18}F]**6g** to imaging TG2 activity, because of the fact that a region of interest was drawn over the full tumour volume in the analysis. Nonetheless, based on the imaging results [^{18}F]**6g** appears to be a successful TG2 PET tracer for imaging of TG2 activity *in vivo*.

6.2 General discussion

The work described in this thesis concerns the development of PET tracers for imaging TG2 activity *in vivo*. TG2 has been implicated in several diseases and various classes of inhibitors have been developed. However, at the start of this study, to the best of our knowledge, no PET tracers for TG2 were available. Only the radiosynthesis of a fluorine-18 labelled peptidic acyl donor substrate had been described, but without purification, isolation and further evaluation. During the present work, fluorine-18 labelled amine acyl acceptor substrates for imaging TG2 activity were reported in conference abstracts. The present studies focused on radiolabeling of potent TG2 inhibitors, which, because of the limited availability of potent compounds, inevitably were irreversible inhibitors. Selection criteria for these inhibitors were affinity in the nanomolar range, selectivity over other transglutaminase family members and stability towards other naturally present thiols such as glutathione. In addition, the ability of these inhibitors to enter the brain by means of passive diffusion over the blood-brain barrier was preferred so that

developed PET tracers would also be available for brain research. For TG2 inhibitors described in literature, no data on blood-brain permeability was available and therefore, compounds were selected on low molecular weights and high lipophilicity.

The compounds described in **Chapter 3** met most of these criteria. All three had reported two-digit nanomolar IC_{50} values (10, 62 and 14 nM for **1**, **2** and **3**, respectively), good selectivity over other transglutaminase family members and high stability towards thiols as glutathione. Furthermore, these compounds were designed for the treatment of Huntington's disease, where inhibition of intracellular TG2 in the brain was pursued, and as such brain uptake of these compounds could be expected. In contrast, however, all three compounds were susceptible to oxidative metabolism as measured by liver microsomal assays and they also appeared to be strong substrates for the efflux transporter P-gp. In addition, compound **1** displayed a drastic drop in potency in cell lysate assays compared with isolated TG2 inhibition assays. For PET imaging, long term metabolic stability is less important and therapeutic windows are of no concern. Brain uptake would be preferred, but at the same time was not essential, as TG2 crosslinking activity is implicated in many diseases in the peripheral system. Furthermore, efflux transporters can be pharmacologically blocked in laboratory animals when required, potentially allowing brain imaging studies. Therefore, radiolabelling and *in vivo* evaluation of these potent TG2 inhibitors was pursued. Indeed, low brain uptake was observed in healthy rats, possibly due to P-gp substrate behaviour. Furthermore, all three compounds were metabolized extensively.

Z006 is a potent TG2 inhibitor that often has been used as a biochemical tool to inhibit TG2 in various *in vitro* assays. This inhibitor has high affinity for TG2, displays high selectivity towards other transglutaminases and does not inhibit caspase-3, a cysteine protease, activity. Structural modification of this tetrapeptidic inhibitor on the N-terminus is tolerated, as was demonstrated by the rhodamine-**Z006** conjugate.

The two most potent inhibitors in our set of **Z006** analogues, **6f** and **6g**, were radiolabelled (**Chapter 4**). Following characterization of the main metabolite of [^{18}F]**6g**, referred to as [^{18}F]**M1**, and establishing that **M1** still is a potent TG2 inhibitor, studies were continued using [^{18}F]**6g**. It should be noted, however, that the *in vivo* signal will be due to an unknown mixture of [^{18}F]**6g** and [^{18}F]**M1**, which clearly will make quantification difficult. Unfortunately, the direct radiosynthesis of [^{18}F]**M1** as an alternative to [^{18F}]**6g** seems unlikely, as the synthesis of [^{18}F]**6g** itself is already laborious and the synthesis of [^{18}F]**M1** would require at least one additional synthetic step. Alternatively, analogues of [^{18}F]**6g** where the C-terminal methyl ester is replaced by a more inert functionality could be designed. It is anticipated, however, that [^{18}F]**M1**, carrying a free carboxylic acid, will not be able to pass biological barriers such as cell membranes and the blood-brain barrier. This could be an advantage when aiming at imaging of extracellular active TG2. Apart from increasing the amount of tracer available for targeting, wash-out of unbound radiotracer could be accelerated when labelled compounds are not residing in intracellular compartments.

The MDA-MB-231 tumour xenograft model used for validation of the selected TG2 PET tracers *in vivo* appears to be a suitable model (**Chapter 5**). TG2 is expressed in the tumour tissue and the PET imaging results using the fluorine-18 labelled **Z006** analogue implicate that administration of tissue transglutaminase inhibitors results in a decrease of tracer uptake in the tumour. The lack of a significant decrease in tumour uptake using **ERW1041E** as TG2 inhibitor might be explained by the limited potency of this inhibitor (K_i = 11 µM). In contrast, tracer uptake in the tumour was significantly decreased by administration of this tracer at low specific activity. This latter result must, however, be interpreted with care, because off-target binding will equally be inhibited when using the same compound for imaging as for blocking. Although more research is required to validate this compound as a successful TG2 PET tracer, initial results imply successful targeting of active TG2 *in vivo*.

6.3 Future perspectives

Based on the successful study of [^{18}F]**6g** in MDA-MB-231 tumour bearing mice, this compound can be used to assess the inhibitory potency of other TG2 inhibitors *in vivo*. To date, *in vivo* behaviour of TG2 inhibitors remains largely unknown and inhibitor optimization is achieved by performing *in vitro* inhibition assays against recombinant purified TG2. Obviously, *in vitro* potency does not necessarily reflect *in vivo* behaviour, where other factors such as bioavailability, metabolism and clearance can be confounding factors. Therefore, the TG2 inhibitor mediated reduction in tumour uptake of [^{18}F]**6g**, could provide a measure of *in vivo* inhibitory potency of novel TG2 inhibitors, resulting in efficient development of future TG2 drug candidates.

However, despite the successful TG2 targeting of [^{18}F]**6g** (**Chapter 5**) in the MDA-MB-231 tumour xenograft model, there are several factors that limit the broad applicability of this compound. First, clearance of the tracer from blood is rather slow, resulting in high background values, especially in well perfused tissues. Second, rapid metabolism of [^{18}F]**6g** to [^{18}F]**M1** will not only hamper quantification of the PET signal, but likely also prevent passive diffusion of this negatively charged metabolite to the intracellular environment, which means that this compound will only (or mainly) measure extracellular TG2 activity. Third, for *in vivo* studies of TG2 activity in organs such as brain, kidneys, liver or intestines, [^{18}F]**6g** appears unsuitable, because of either low uptake (brain) or high uptake in these organs. Fourth, the tedious synthesis lasting over 2.5 hours and involving four challenging radiochemical steps will challenge its availability in many centres.

Zedira GmbH, a biotech company specialized in transglutaminase research, recently started Phase I clinical trials with a new TG2 inhibitor, **ZED1227**, with the ultimate goal of finding a therapy for treatment of celiac disease. This compound (figure 3)

can be regarded as a peptidomimetic analogue of **Z006**, which was also developed by Zedira GmbH. This newly developed compound has an excellent inhibitory profile against other transglutaminase family members, good stability in a liver microsome assay, and high metabolic stability in gastric and intestinal juices. Structurally, this peptidomimetic compound carries a 2-ethylbutylamide on the 'C-terminus' instead of the labile C-terminal methyl ester present in **Z006**. The diazoketone functionalty has been replaced with an acrylic acid Michael acceptor functionality. The lack of a diazoketone structure, a challenging functionality in the course of the present studies, will increase the (radio)synthetic flexibility. **ZED1227** could be labelled with carbon-11 at the 'N-terminal' methyl position of the imidazole group or methyl ester functionality. Alternatively, analogues amenable to fluorine-18 could be developed. Then, the fluorobenzylidene-oxime structure, analogous to [^{18}F]**6g**, would be a promising starting point.

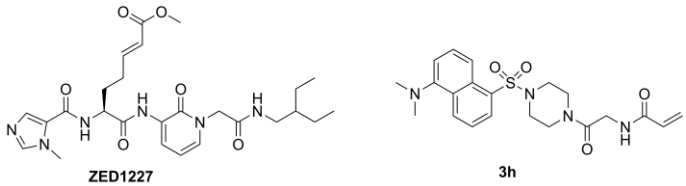

Figure 3: Promising TG2 inhibitors that allow for translation to PET tracers

Another interesting small molecule TG2 inhibitor, **3h**, was published by Griffin and coworkers in 2015 (figure 3). Carrying an acrylamide functionality, radiolabeling by aminocarbonylation as described in **Chapter 3** is easily feasible. Unlike most TG2 inhibitors, this compound has been thoroughly evaluated *in vitro* and *in vivo*. For example, this compound was shown to be cell permeable, allowing for intracellular imaging of TG2 activity. In addition, *in vivo*, in a mouse model of hypertensive nephrosclerosis the continuous subcutaneous administration of **3h** resulted in a significant decrease in collagen deposition, a measure of kidney scarring.

In a similar animal model, a TG2 inhibiting antibody was shown to be effective as well in reducing kidney scarring. Potentially, antibodies with such characteristics, *i.e.* the apparent inhibition of TG2 crosslinking activity, could be used as a basis for TG2 imaging by means of so-called 'immuno-PET'. Such high molecular weight constructs are not excreted via the kidneys and thus allow for PET studies involving subjects with renal TG2 overactivity. As briefly discussed in **chapter 2**, radiochemistry towards radiolabelled antibodies using for example zirconium-89, has been well described and as such, labelled antibodies should be easily accessible.

As discussed in **Chapter 2**, it is unlikely that reversible inhibitors with affinities in the range required for successful PET imaging are currently available. Potent reversible inhibitors are by us suspected from irreversible binding and lack of selectivity. To avoid inconclusive results in TG2 inhibitor development in future studies, it is strongly recommended that results from *in vitro* binding experiments using isolated recombinant transglutaminase are supported with inhibition experiments on more complex systems (e.g. tissue sections, cell lysates).

In contrast, radiolabelled acyl donor substrate peptides are available and are currently explored for imaging TG2 activity. The use of these constructs, due to their size, will be limited to determination of extracellular TG2 activity. A similar substrate strategy was applied previously for imaging enzymatic activity of FXIIIa, a member of the transglutaminase family involved in blood coagulation, however, with limited success. The fact that the latest report dates from 2006 might give rise to concerns on the viability of this strategy.

Overall, PET could play a major role in TG2 drug development for several reasons. First of all, because of difficulties in correlating *in vitro* TG2 expression with *in vivo* TG2 crosslinking activity, a biological tool that allows for *in vivo* TG2 activity measurements is required. Secondly, *in vivo* assays using unlabelled TG2 inhibitors

for direct determination of inhibitory potency are available. Commonly, TG2 mediated inhibition of induced renal scarring in mice is used as a measure of inhibitor potency, which has the obvious limitation that test animals need to be sacrificed for immunohistochemical detection of collagen deposits as a marker of TG2 activity. Use of a PET tracer allows for direct evaluation of TG2 activity and thus provides a direct measure of drug potency. Furthermore, as PET is a quantitative imaging technique, optimal drug dosage can be determined. Finally, because of the non-invasive nature of PET, longitudinal studies in the same subject can result in rapid and efficient determination of TG2 drug effects.

Taken together, the availability of a well validated PET tracer for imaging TG2 activity is of great importance for efficient drug development. The current work has resulted in the first PET tracer that has shown to target active TG2 *in vivo* and provides directions and suggestions for future TG2 PET tracer development. It is therefore anticipated that in the near future novel TG2 PET tracers with improved characteristics will be developed to aid in TG2 drug development.